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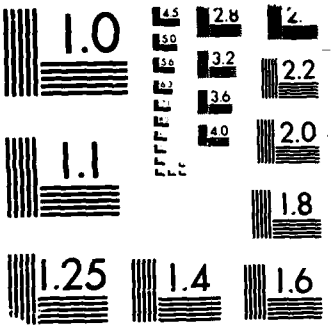

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)
Trypsin-like and chymotrypsin-like enzymes have been identified and separated from the digestive tracts of three model insects: the rust red flour bettle Tribolium castaneum, the mealworm Tenebrio molitor and the locust Locusta migratoria. These trypsins and chymotrypsins can be fully inhibited by the proteinaceous trypsin-chymotrypsin inhibitors from legume seeds, such as the Bowman-Birk inhibitor (BBI) from soybeans and CI from chickpeas. The purified and partially-characterized insect

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FINAL REPORT

(FIRST ANNUAL REPORT)

3RD INTERIM

PROTEASES OF STORED PRODUCT INSECTS AND THEIR INHIBITION BY SPECIFIC PROTEASE INHIBITORS FROM SOYBEANS AND WHEAT GRAIN

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CONTENTS

STATEMENT OF THE PROBLEM STUDIED

Specific protease inhibitors, which inhibit digestive proteases of insects, have evolved in plants. These inhibitors are of potential interest in protection of valuable crops from damage by insects. It has been the objective of the present study to isolate and characterize digestive proteases of several model insect pests and to investigate their naturally-occurring inhibitors from legume seeds and grains.

SUMMARY OF MOST IMPORTANT RESULTS

The separation and partial characterization of trypsin and chymotrypsin-like enzymes from the digestive tracts of the insects Tenebrio molitor, Tribolium castaneum and Locusta migratoria and the inhibitability of these enzymes by proteinaceous trypsin-chymotrypsin inhibitors from soybeans and chick peas suggest that these insects may be affected by the inhibitors in vivo.

The lack of disulfide bridges in the insects proteases suggest a difference in their conformation and assembly from that known for the respective mammalian enzymes. Further investigation of the structure of insect proteases may lead to better understanding of their susceptibility to inhibitors in vivo.

(ISPAEL)

PARTICIPATING SCIENTIFIC PERSONNEL

Dr. P. SMIRNOFF
Isolation and modification of protein proteinase inhibitors

R. GOLAN*
M.Sc. Thesis:

Isolation, characterization and comparative study of proteolytic enzymes from the midguts of Tenebrio molitor adults and larvae as a basis for possible biological pest-control with naturally occurring protease inhibitors from plants sources.

E. SAKAL* M.Sc. (graduate student)
Isolation and characterization of locust proteinases

N. YONAH* (graduate student)
Isolation and characterization of Tribolium proteinases

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*No charge to grant

PREFACE

Protein protease inhibitors of plant origin are of potential interest in protection of valuable crops from damage as a consequence of attack by insects. The investigation of the digestive proteases of several model insects is a pre-requisite for understanding the complex relationships that the insects have evolved with the plant. The information on insect proteolytic enzymes and more specificially, on insect trypsins and chymotrypsins, in comparison to the corresponding proteases from higher organisms, is essential for studying the selective interactions of the naturally-occurring protease inhibitors with the insect proteases.

While mammalian pancreatic trypsins and chymotrypsins are well-known enzymes that are present throughout the vertebrates, much less is known about trypsins and chymotrypsins from insects. In general, trypsins and chymotrypsins of insects are characterized on the basis of their substrate specificity and their inhibitability by specific inhibitors. These criteria are also being used throughout our studies.

The research performed during November 1986 - November 1987 was primarily devoted to the isolation and characterization of digestive proteases of three model insects. The detection, preliminary purification and characterization of digestive proteases of the rust red flour beetle (Tribolium castaneum), the mealworm (Tenebrio molitor) and the locust (Locusta migratoria) - a major pest of crops in Third World countries - were briefed in the First Interim Report. The present report deals with further purification and characterization of the latter. Although strong proteolytic activity has been demonstrated in the midgut lumen of the locust, very little is known concerning the biochemistry of its digestive system. We also describe the relative effectiveness of a series of synthetic and naturally-occurring inhibitors of mammalian serine proteases in blocking the insect enzymes.

EXPERIMENTAL AND RESULTS

(1) Tenebrio molitor proteases

1.a Extraction, Separation and Isolation

Our earlier experiments showed that trypsin, chymotrypsin and carboxypeptidase B are major proteases in the digestive track of Tenebrio molitor larvae and adults. Preliminary experiments with different parts of the guts indicated that most of the Tenebrio trypsin and chymotrypsin is concentrated in the midgut. The following results relate to the isolation and characterization of trypsin and chymotrypsin from the alimentary system of Tenebrio molitor adults.

Tenebrio molitor larvae and adults were reared in glass jars on a wheat-bran diet, at 24 - 26°C. Humidity was maintained by covering the medium with moist cotton wool. Adults were dissected at the rectal region and the midguts were extruded and disconnected from the head. The detached midgut was cleared of fat-body. Midgut homogenates were prepared in a chilled tissue grinder containing distilled water at 5°C. The homogenates were centrifuged in the cold and the supernatant enzyme solution was decanted and utilized for isolation of proteases. The isolation of Tenebrio adult trypsin and chymotrypsin was achieved by column-chromatography on the anion exchanger DEAE-cellulose followed by affinity chromatography on p-aminobenzamidine (PABA)-Sepharose and phenylbutylamine-Sepharose (PBA), respectively. The purification of Tenebrio adult trypsin is shown in Fig. 1).

1.b. Electrophoretic properties and molecular weight

Tenebrio trypsin and Tenebrio chymotrypsin showed single homogenous bands upon electrophoresis on cellulose acetate membranes at pH 7.3 and on polyacrylamide gels at pH 4.5.

The molecular weight of Tenebrio trypsin was determined as 16700 by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and as 16500 by exclusion chromatograpphy on a Sephadex-G-50 column. The molecular weight of Tenebrio chymotrypsin was estimated from amino acid composition as 16400. The relatively lower molecular weights of Tenebrio trypsin and chymotrypsin are remarkable. No zymogens of these enzymes have been found thus far.

1.c. Amino acid composition

The amino acid composition of Tenebrio trypsin and chymotrypsin, as compared to trypsins and chymotrypsins from other sources, are given in Table 1. They clearly demonstrate that trypsin and chymotrypsin from Tenebrio adults differ in amino acid compositon from the respective enzymes of Tenebrio larvae and from the bovine enzymes. The complete lack of disulfide bonds in the Tenebrio adult proteases as compared to the six S-S bonds in bovine trypsin, suggests a significant difference in conformation.

1.d. Kinetic properties of Tenebrio trypsin

The kinetic properites of Tenebrio trypsin (from adults and larvae) were determined with the specific synthetic substrates tosyl-L-arginine methyl ester (TAME) and N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) and with casein and were compared to bovine trypsin at pH $\sim$ 8. Significant differences, varying from substrate to substrate, were noted between the three trypsins. Similar variations were noted for Tenebrio chymotrypsin with respect to the specific synthetic substrates N-acetyl-L-tyrosin ethyl ester (ATEE) and N-acetyl-DL-tyrosine-p-nitroanilide (ATPNA) and to casein.

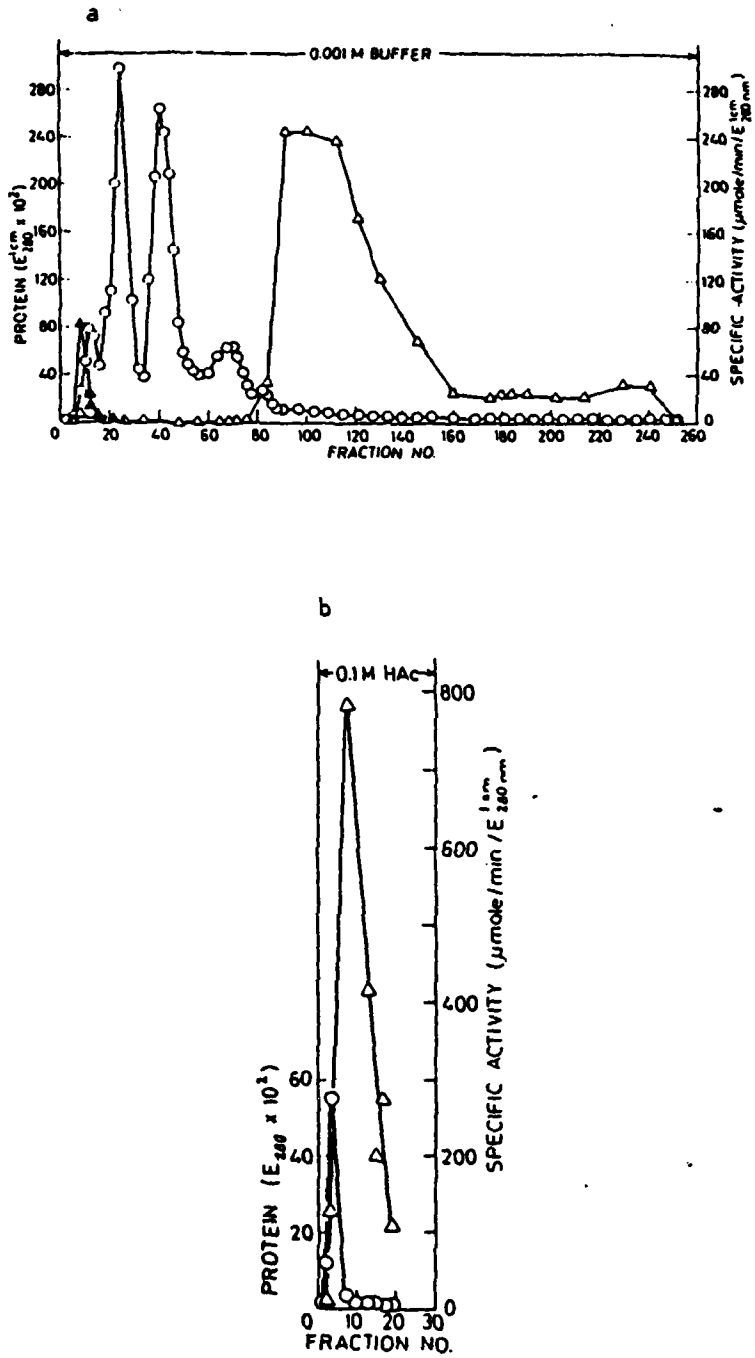


Fig. 1: Elution profile for the purification of the trypsin-like enzyme from the midguts of Tenebrio molitor adults by successive column chromatography.

(a) On DEAE-cellulose equilibrated with ammonium acetate buffer at pH 6.8.

(b) Affinity-chromatography of the active trypsin (fractions 85 - 145) from the DEAE-cellulose column on a PABA-Sepharose column, applied with Tris-HCl buffer at pH 8 and displaced with acetic acid.

o - Protein ($E_{280\,nm}$)
Δ - Tenebrio trypsin-like activity
▲ - Tenebrio chymotrypsin-like activity

COMPARISON OF AMINO ACID COMPOSITION OF TRYPSINS AND CHYMOTRYPSINS FROM DIFFERENT SOURCES

	TRYPSIN					CHYMOTRYPSIN		
				LOCUST				
AMINO ACID	Tenebrio molitor larvae[1]	Tenebrio molitor adults	BOVINE[2]	$TLE_{Aff.1}$	$TLE_{Aff.2}$	Tenebrio molitor larvae	Tenebrio molitor adults	BOVINE[3]
LYSINE	3	4	14	3	3	6	16	14
HISTIDINE	3	3	3	5	9	3	2	2
ARGININE	6	4	2	4	8	7	8	4
ASPARTIC ACID	16	23	22	17	17	20	15	23
THREONINE	13	10	10	15	19	12	10	23
SERINE	33	14	33	16	26	14	13	28
GLUTAMIN ACID	13	18	14	21	23	22	13	15
PROLINE	8	8	9	11	6	10	11	9
GLYCINE	30	19	25	27	30	21	15	23
ALANINE	16	13	14	14	22	19	11	22
HALF CYSTINE	4	0	12	2	0	8	1	10
VALINE	19	13	17	13	18	15	11	23
METHIONINE	1	0	2	2	-3	2	1	2
ISOLEUCINE	12	9	15	9	11	8	8	10
LEUCINE	13	16	14	11	14	12	11	19
TYROSINE	8	4	10	7	11	5	3	4
PHENYLALANINE	1	4	3	4	4	7	4	6
TRYPTOPHAN	0	8	4	*	5	*	*	8
TOTAL	199	171	223	181	229	191	153	245
MOLECULAR WEIGHT	19 768	18 382	23 800	21 720	27 850	19 622	16 380	25 600

*Not determined

[1]Levinsky, H., Birk, Y. & Applebaum, S.W. (1977) Int. J. Peptide Protein Res. 10:252

[2]Walsh, K.A. & Neurath, H. (1964) Proc. Nat. Acad. Sci. U.S.A. 52:884

[3]Blow, D.M., Birktoft, J.J. & Hartley, B.S. (1969) Nature 221:337

1.e. Effect of inhibitors on Tenebrio trypsin

Tenebrio adult trypsin - in a similar manner to bovine trypsin - were inhibited at a 1:1 molar ratio by the naturally-occurring, proteinaceous, trypsin inhibitors BBI from soybeans and CI from chick peas, when assayed on casein and on the specific synthetic substrates TAME and BAPNA.

(2) Tribolium castaneum proteases

As described in the First Interim Report, insect cultures of Tribolium castaneum larvae have been maintained at 32°C on commercial white wheat flour supplemented with 5% brewers yeast. Larval midguts of last instar larvae have been used for preparation of aqueous midgut enzyme extracts. The latter exhibited pronounced trypsin- and chymotrypsin-like activities when assayed on the specific synthetic substrates TAME and BAPNA for trypsin and ATEE and ATPNA for chymotrypsin. These activities have been fully inhibited by the chloromethyl ketones TLCK and TPCK which are specific active site titrants of trypsin and chymotrypsin, respectively. Moreover, they were also fully inhibited by the trypsin - chymotrypsin inhibitors BBI from soybeans and CI from chickpeas. Separation and purification of these enzymes by gel filtration followed by ion-exchange HPLC is now in progress.

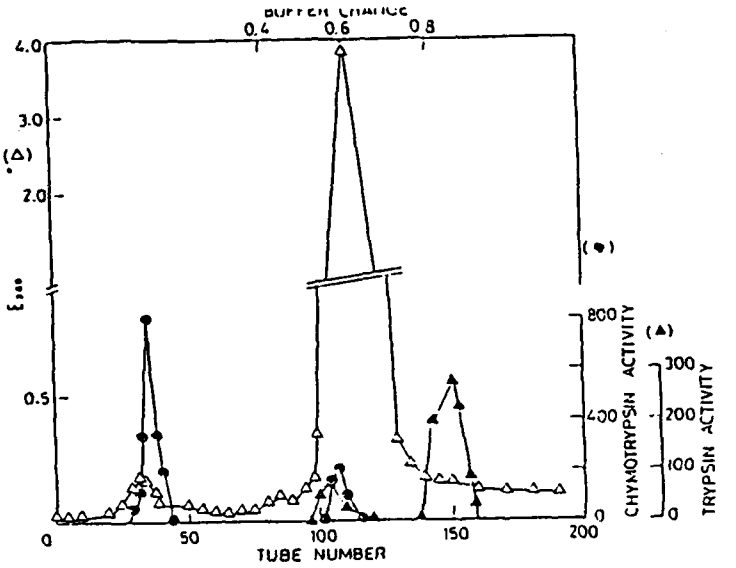
(3) Locust proteases

3.a. Extraction, Separation and Isolation

Locusts (Locusta migratoria) were reared at 28 - 30° on grass and oatmeal. The starting material for the isolation of the digestive proteases was an aqueous extract of the caecae, which were found to be the optimal source for the proteases. Primary purification was carried out by ion exchange chromatography on a DEAE-cellulose column, in which the two trypsins appeared in the anionic fraction and the chymotrypsin in the cationic fraction (Fig. 2). Further purification and full separation of the locust trypsins was achieved by affinity chromatography on a PABA-Sepharose column. The two trypsins were designated $TLE_{Aff.1}$ and $TLE_{Aff.2}$. The major trypsin-like enzyme, $TLE_{Aff.2}$, served as the main source of trypsin for this research.

3.b. Electrophoretic properties, molecular weight and amino acid composition of locust trypsins

The purity and the homogeneity of the enzymes were demonstrated by electrophoresis on cellulose acetate strips and polyacrylamide gels with and without SDS. The molecular weights of $TLE_{Aff.1}$ and $TLE_{Aff.2}$ were determined by SDS-PAGE as 17000 daltons and 24000 daltons, respectively. The amino acid compositions of the locust trypsins were found to be similar to those of adult Tenebrio trypsin and chymotrypsin , which are characterized by the lack or low content of sulfur-containing amino acids in general and of half cystines in particular (Table 1).



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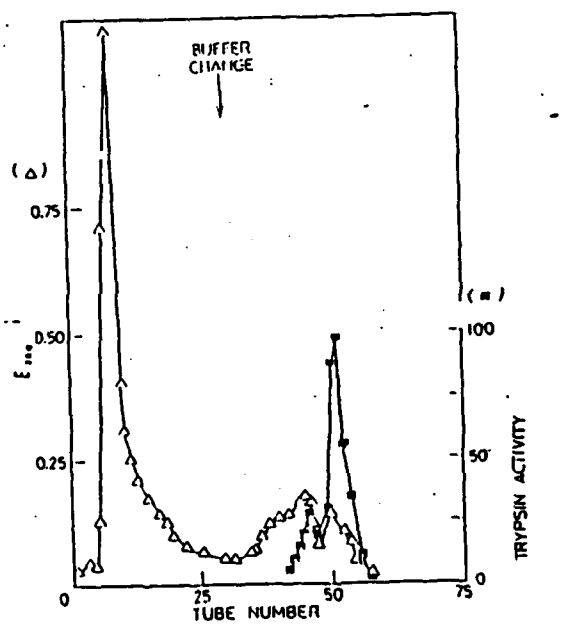

Fig. 2: Elution profile for the purification of the trypsin-like enzyme from the caecae of the locust Locusta migratoria by successive column chromatography.

(a) On DEAE-cellulose equilibrated with 0.01 M Tris-HCl buffer pH 8 and eluted with stepwise increases (0.4 M, 0.6 M and 0.8 M) of NaCl in the same buffer.

Δ - Protein (E_{200} nm)
▲ - Locust trypsin-like activity
● - Locust chymotrypsin-like activity

(b) Affinity-chromatography of the active trypsin (tubes 100 - 130) from the DEAE-cellulose column on a PABA-Sepharose column applied with Tris-HCl buffer at pH 8 and displaced with 6 M urea in the same buffer.

Δ - Protein (E_{200} nm)
■ - Locust trypsin-like activity

3.c. Kinetic properties of locust trypsin

$TLE_{Aff.2}$ was highly active on specific substrates for trypsin. The Km and Kcat values, determined on esterolytic, amidolytic and proteolytic substrates, were similar to those of the respective bovine enzymes. Activation of substrate, a phenomenon known for the reaction of bovine trypsin and TAME, was also observed for $TLE_{Aff.2}$.

3.d. Effect of inhibitors

$TLE_{Aff.2}$ was inhibited by naturally-occurring trypsin inhibitors, such as BBI (the Bowman-Birk Inhibitor) and STI (the Kunitz inhibitor) from Soybeans, CI (chickpea inhibitor), COM (chicken ovomucoid) and TOM (turkey ovomucoid). However, STI was found to be a less potent inhibitor of $TLE_{Aff.2}$ than of bovine trypsin. Otherwise, the Ki values of $TLE_{Aff.2}$ were similar to those of the respective bovine enzymes.

Locust trypsin and chymotrypsin were inactivated by phenylmethylsulfonyl fluoride (PMSF) and by specific chloromethylketones, indicating the involvement of serine and histidine in their active sites.

The above findings indicate similarities between the trypsins of the locust Locusta migratoria and the mammalian trypsin in kinetic parameters, in inhibition features and in molecular weight. Differences were found in amino acid composition, especially regarding S-S bonds, which point towards conformational differences between the locust and bovine trypsins. No zymogens of Locust trypsin or chymotrypsin have been found thus far.

(4) Proteinase inhibitors from seeds

In order to study the interaction of the naturally-occurring proteinase inhibitors with the insect proteinases, we have prepared a series of native and modified double-headed trypsin-chymotrypsin inhibitors from soybeans (BBI) and from chickpeas (CI). These include cleavage of BBI and CI by cyanogen bromide followed by pepsin, which results in complete separation between the trypsin- and chymotrypsin-inhibiting domains of the inhibitors. In addition, photoaffinity labeled inhibitors (with 2-nitro-4(5)-azido phenyl-sulfenyl chloride) were prepared and will be used in due time in the attempts to bind the inhibitors covalently to the respective enzymes. Such studies, if successful should shed light on the localization of the proteases in the digestive tract.

Similar experiments with the specific Tribolium proteinase inhibitor from soybeans are in progress.

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